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TITLE: An in Vivo shRNA-Drug Screen to Identify Novel Targeted Therapy Combinations for KRAS Mutant Cancers

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14. ABSTRACT

KRAS mutations occur in ~90% of all pancreatic cancers and in ~20% of all human cancers, but no effective therapies targeting KRAS have been developed. We proposed a functional genomic screen to identify gene targets that, when inhibited, cooperate with MEK inhibitors (which block signaling through the MAPK pathway, a key KRAS effector) to kill KRAS mutant pancreatic cancer cells in order to develop novel therapeutic combinations for these cancers. Our efforts identified several components within the MAPK pathway that can lead to feedback reactivation of MAPK signaling despite the presence of MEK inhibitors, creating a key vulnerability of MEK inhibitors that can lead to resistance. Importantly, we found that ERK inhibitors (which block downstream of MEK inhibitors) could overcome MAPK reactivation and produced enhanced efficacy. Our efforts also identified multiple members of a metabolic and autophagy-regulating pathway, suggesting that autophagy inhibitors (currently in clinical trials for pancreatic cancer) in combination with MAPK inhibitors may be a promising approach. We expect these data to drive new clinical trials for pancreatic cancer patients.

15. SUBJECT TERMS
KRAS mutation, pancreatic cancer, MEK inhibitor

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INTRODUCTION:

Oncogenic KRAS mutations are found in ~90% of pancreatic ductal adenocarcinoma (PDAC) and ~20% of all human cancers. However, to date, efforts to develop inhibitors that target KRAS directly have been unsuccessful. One alternative strategy has been to target instead downstream effectors of KRAS, either alone or in combination. Large-scale screening of cancer cell lines with libraries of targeted inhibitors revealed that the most effective class of agents in KRAS mutant PDAC cell lines were MEK inhibitors, which block signaling through the MAPK pathway—a key effector pathway activated by KRAS. Clinical trials of MEK inhibitors in PDAC patients have shown high rates of disease stabilization, but few true tumor responses were noted(*1*). These findings suggest MEK inhibitors may be promising backbones for targeted therapy combination strategies for KRAS mutant PDAC. Large-scale functional genomic or "synthetic lethal" RNAi screens represent a potentially powerful tool for identifying novel gene targets for cancer therapy, but have two major weaknesses: (1) Most RNAi screens assess the effect of RNAi-mediated gene inhibition alone and have not been leveraged to identify potential combination therapies, a promising emerging clinical approach, and (2) RNAi screens are typically conducted *in vitro*, and do not address the effects of the *in vivo* tumor microenvironment and do not necessarily select for those targets most likely to produce the dramatic *in vivo* responses needed for clinical efficacy in patients. To address these deficiencies, we attempted to develop a novel *in vivo* RNAi-drug screen approach utilizing mouse models of PDAC. The goal of this study was to identify novel gene targets that, when inhibited, cooperate with MEK inhibitors to kill KRAS mutant PDAC cells in order to develop new and effective targeted therapy combinations for PDAC patients.

KEYWORDS:

KRAS mutation

Pancreatic cancer

MEK inhibitor

OVERALL PROJECT SUMMARY:

Summary of Progress by Specific Task

<u>Task 1:</u>

Following approval of the appropriate animal protocol (*task 1a*), a primary *in vivo* shRNA-drug screen in mouse PDAC xenografts was attempted using 6 PDAC cell lines (*tasks 1b,c*). Unfortunately, technical limitations restricted our ability to obtain workable data from the pooled shRNA-drug screen in xenografts. The major factor that limited our ability to successfully execute the screen in xenografts was the efficiency with which these PDAC cell lines formed subcutaneous xenograft tumors. In order to achieve the desired coverage of at least 1000 cells per shRNA in the library, injection of 5-10 million cells into each mouse was required. However, for most cell lines, only a fraction of the cells injected survive to form a tumor. Thus, the surviving fraction of cells proved insufficient to provide the necessary ratio of cells to shRNA required to generate quality shRNA screen data. Multiple efforts to troubleshoot the process were undertaken, including conducting parallel screens using standard approaches in these PDAC cell lines as a control. Still, despite multiple attempts, suitable *in vivo* screening conditions could not be established. Overall, we concluded that, while an *in vivo* screening strategy may hold potential benefits, the feasibility of a large-scale *in vivo* screening strategy in PDAC with presently available technologies is limited.

<u>Task 2:</u>

Despite the technical difficulties experienced with Task 1, an shRNA “mini-pool” was successfully constructed (*task 2a*) for prioritization and validation of candidate targets based on top hits identified through parallel control screens conducted in Task 1 using standard methods. However, the same technical issues related to the required efficiency of tumor formation for adequate shRNA representation also limited our attempts to successfully execute the secondary orthotopic shRNA-drug screen (*tasks 2b,c*). Again, despite efforts to troubleshoot the process, including parallel control validation experiments performed using standard methods, we were not able to establish suitable in vivo screening conditions. However, through our parallel control experimental efforts we were able to validate and prioritize two top-tier hits for further characterization and *in vivo* efficacy evaluation in mice in Task 3.

<u>Task 3:</u>

While the technical difficulties experienced in performing Tasks 1 and 2 created significant delays in the project, we were ultimately able to initiate *in vivo* testing of potential therapeutic strategies in a mouse PDAC model, as originally proposed, based on hits prioritized in Task 2. Although the data are not mature, a MEK inhibitor and ERK inhibitor (based on prioritized hits) are currently being evaluated for efficacy (*task 3a*) and pharmacodynamic effect (*task 3b*), with a particular focus on the ability to achieve robust and sustained suppression of MAPK signaling (as described in *Summary of Findings and Potential Impact* below). For pharmacodynamic experiments, as originally proposed, both a short (3 day) and longer (4 week) timepoint will be assessed to evaluate the degree of MAPK suppression achieved upon initiation of therapy and the ability to produce sustained pathway suppression and to prevent feedback reactivation of the pathway during prolonged therapy. Analogous studies using inhibitors of key metabolic pathways (based on additional prioritized hits) in combination with a MEK inhibitor are planned for initiation in the near future.

Summary of Findings and Potential Impact

Interestingly, all three RAF family genes were among the top five hits identified through our screening efforts. This finding is somewhat surprising since RAF kinases act upstream of MEK in the MAPK pathway, and mediate MEK phosphorylation and activation. Thus, it might be expected that MEK inhibitors would not be affected by RAF kinase activity, since they block the kinase cascade at a point downstream of RAF. Furthermore, the initial expectation would be that this approach would identify targets outside the MAPK pathway that cooperate with MAPK blockade exerted by MEK inhibitors. However, hyperactivation of MEK by RAF proteins has previously been shown to lead to resistance to MEK inhibitors by abrogating the ability of MEK inhibitors to suppress MAPK signaling(*2, 3*). Therefore, this finding suggested that perhaps feedback reactivation of MAPK signaling through enhanced activity of RAF kinases could be a major limitation on the efficacy of MEK inhibitors. Indeed, we found that following prolonged treatment with MEK inhibitors, MAPK pathway signaling became reactivated in KRAS mutant PDAC cells despite continued presence of MEK inhibitor (**Figure 1**). MAPK pathway reactivation was not due to degradation of drug, as fresh drug was added every 24 hours during treatment and one hour before lysis. Rather, it appeared that feedback signaling leads to increased activation of RAF activity (evidenced by increased phosphorylation of CRAF (P-CRAF) and increased phosphorylation and hyperactivation of MEK (Figure 1), leading both to a rebound in levels of phosphorylated ERK (P-ERK) and phosphorylated RSK (P-RSK), a key target of ERK activity. Feedback reactivation of MAPK signaling was observed in the presence of two different MEK inhibitors, selumetinib (AZD6244) and the newer generation MEK inhibitor, trametinib, although feedback reactivation was less pronounced in the presence of trametinib. Still, these data suggest that MAPK pathway reactivation and incomplete pathway suppression by MEK inhibitors may be a major factor limiting the activity of these agents.

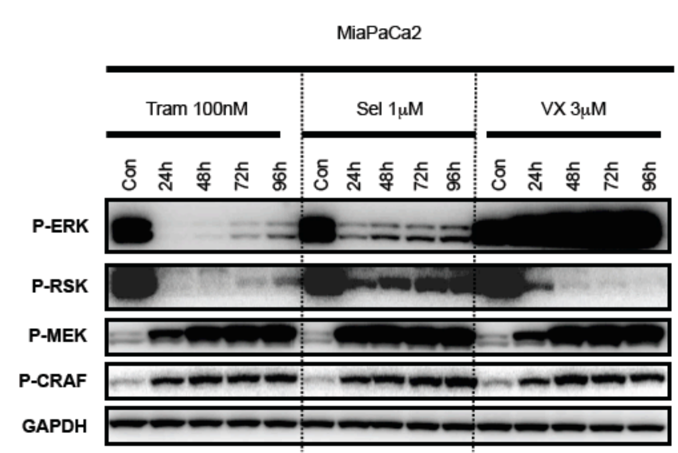

Figure 1: Feedback reactivation of MAPK signaling during prolonged exposure to MEK inhibitors, but not ERK inhibitors. Western blot of KRAS mutant cancer cells treated with the MEK inhibitors trametinib (Tram) or selumetinib (Sel), or the ERK inhibitor VX-11e (VX) for the indicated times. Feedback reactivation of MAPK signaling is evidenced through rebound or P-ERK and P-RSK. As ERK inhibitors increase P-ERK, suppression of MAPK activity is measured by assessment of P-RSK only.

Importantly, however, we found that ERK inhibitors, which inhibit downstream of MEK, were far less susceptible to feedback reactivation of the MAPK pathway and were able to sustain prolonged suppression of MAPK signaling (**Figure 1**). As many ERK inhibitors actually cause an increase in P-ERK levels(*4, 5*), the ability of ERK inhibitors to promote sustained MAPK pathway inhibition is best

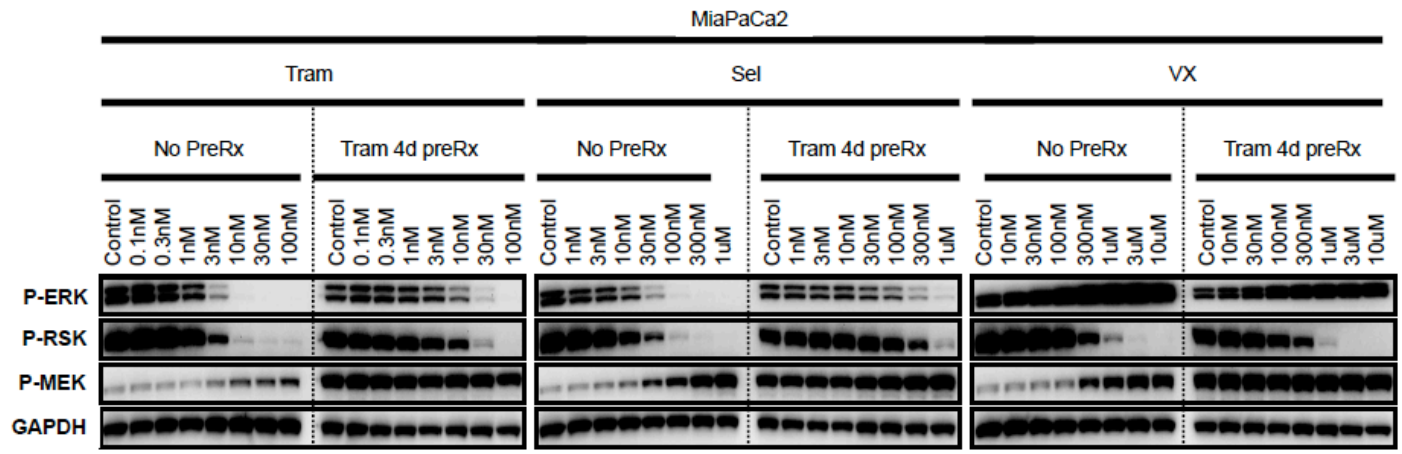

Figure 2: Decreased potency for MAPK inhibition of MEK inhibitors, but not ERK inhibitors, following prolonged exposure of KRAS mutant cancer cells to MEK inhibition. Western blot of KRAS mutant cancer cells without pre-treatment (No preRx) pre-treated for 4d with 100nM trametinib (Tram 4d preRx). Following pre-treatment, drug-containing media was removed and cells were treated for an additional 2 hours with the indicated concentrations of compounds prior to cell lysis. A ~10-30-fold rightward shift is observed in the concentration of MEK inhibitors needed to suppress MAPK signaling (assessed by P-ERK or P-RSK) following trametinib pre-treatment, whereas no shift in potency is observed for the ERK inhibitor VX-11e (VX).

evidenced by maintained suppression of the downstream ERK target P-RSK. Consistent with these findings, we observed that following prolonged exposure of KRAS mutant PDAC cells to MEK inhibitors, the potency with which MEK inhibitors could suppress MAPK signaling was markedly reduced (**Figure 2**). Cells were pre-treated for 4 days with 100nM trametinib and then treated for 2 hours with vehicle or with various concentrations of MEK or ERK inhibitors. After 4 days of MEK inhibitor pre-treatment, the ability of trametinib or selumetinib to suppress P-ERK and P-RSK was reduced by ~10 to 30-fold, suggesting that MEK inhibitors may lose efficacy in KRAS mutant PDAC cells following prolonged exposure. Conversely, the potency with which the ERK inhibitor VX-11e was able to suppress MAPK signaling was unaffected by MEK inhibitor pre-treatment (Figure 3). Collectively, these results suggest that feedback signaling changes occurring after prolonged MEK inhibitor treatment can lead to MAPK reactivation despite the continued presence of drug, but that ERK inhibitors are able to promote sustained MAPK pathway suppression despite these same feedback signaling changes.

Importantly, the ability of ERK inhibitors to maintain continued suppression of MAPK signaling translated into an improved ability to suppress KRAS mutant cell lines in long-term growth assays, compared to MEK inhibitors (**Figure 3**). While the earlier generation MEK inhibitor selumetinib delayed cell growth relative to vehicle control, rapid outgrowth of cells was observed by as little as 1-2

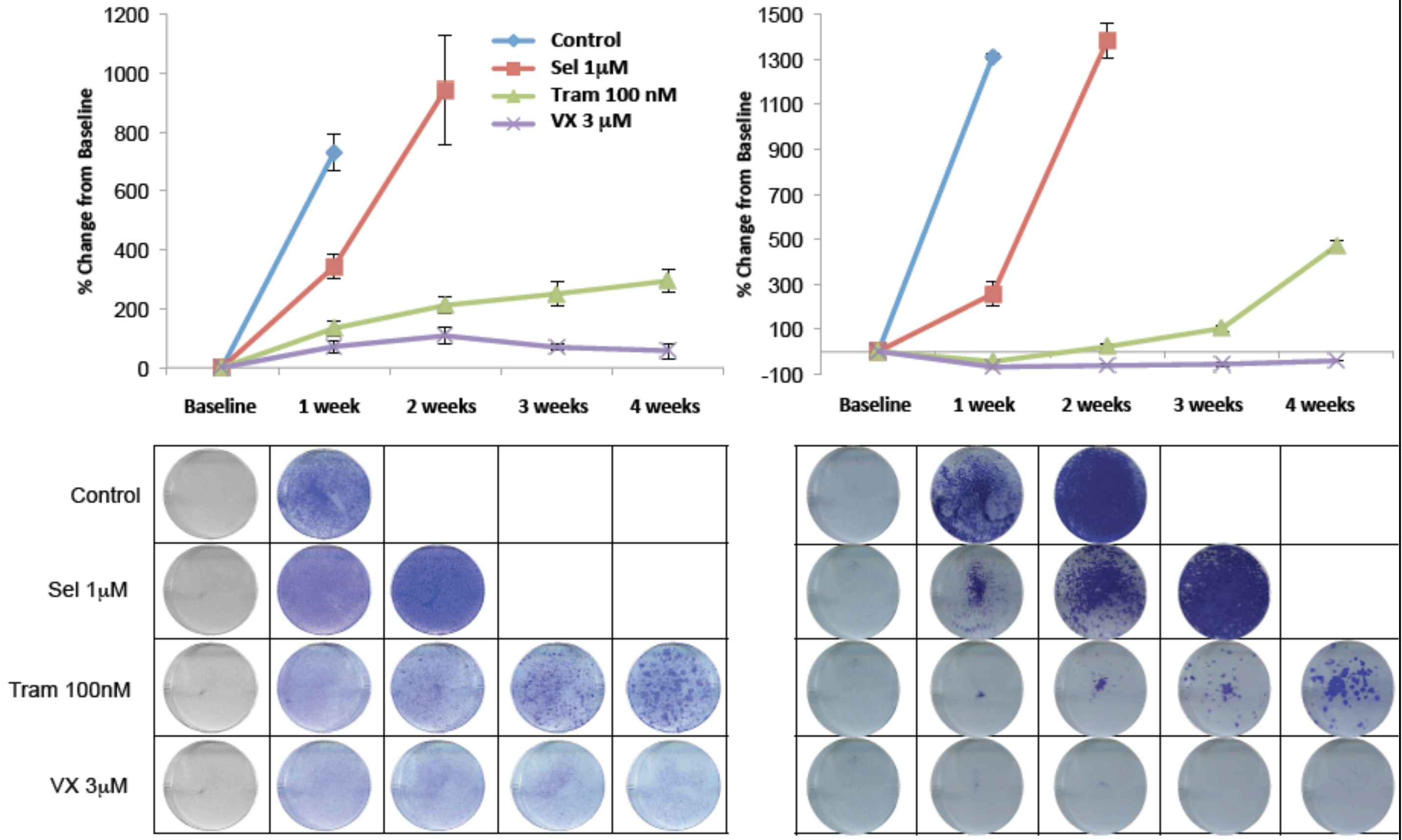

Figure 3: Improved and prolonged suppression of cancer cell growth by ERK inhibitors, compared to MEK inhibitors. Cells were treated for 1-4 weeks with the indicated concentrations of inhibitors, with fresh inhibitor-containing media added every 3-4 days throughout treatment. At each timepoint, parallel plates were stained with crystal violet (bottom panel) and crystal violet staining was quantified (top panel). Eventual outgrowth of tumor cells is seen during prolonged treatment with either MEK inhibitor (selumetinib or trametinib), but the ERK inhibitor VX-11e maintains suppression of cell growth throughout the 4 week treatment period.

weeks, consistent with the rapid and robust reactivation of MAPK signaling observed with prolonged treatment with selumetinib (Figure 1). Although, trametinib showed improved ability to suppress cell growth compared to selumetinib, consistent with the lesser degree of MAPK pathway reactivation observed with trametinib, cell outgrowth was still observed by 3-4 weeks. However, consistent with the ability of ERK inhibitors to maintain prolonged MAPK pathway suppression, the ERK inhibitor VX-11e was able to sustain complete suppression of cell growth and no outgrowth was observed, even by 4 weeks. Currently, the ability of ERK inhibitors to maintain effective MAPK pathway suppression compared to MEK inhibitors in PDAC tumor cells *in vivo* is currently ongoing, as described above for Task 3, and potential differences in the anti-tumor efficacy of these inhibitors is also being compared.

This finding has major implications for targeted therapy strategies for PDAC and other KRAS mutant cancers. Presently, MEK inhibitors are the main class of MAPK pathway inhibitors being evaluated in clinical trials for PDAC and other KRAS mutant cancers and are the backbone for many novel targeted combinations currently in clinical trials. Our data suggests that susceptibility of MEK inhibitors to feedback reactivation of MAPK signaling may be a major factor that can limit the efficacy of MEK inhibitors and any MEK inhibitor-based targeted therapy combinations. ***Our findings***

suggest that ERK inhibitors should be evaluated as potential alternatives to MEK inhibitors for MAPK inhibition in PDAC and other KRAS mutant cancers and perhaps could represent a superior backbone for potential therapeutic combinations for these cancers. Discussions to initiate clinical trials utilizing ERK inhibitor backbones in PDAC or other KRAS mutant cancers are currently underway.

Also among the top hits identified were multiple members of the LKB1-AMPK pathway, which regulates many key cellular metabolic functions, including autophagy (**Figure 4**). Recently, several studies have suggested a unique dependence of PDAC on autophagy(*6*), and autophagy inhibitors are currently in clinical trials in PDAC patients. These data suggest that concomitant blockade of autophagy in combination with MEK inhibition could have a synergistic effect in PDAC. One possible mechanism is that autophagy may be a key compensatory response that allows PDAC cells to survive in the setting of MAPK inhibition by MEK inhibitors. Consistent with this hypothesis, we observed marked induction of autophagy in PDAC cells following treatment with a MEK inhibitor (**Figure 5**). Preclinical studies exploring the therapeutic potential of co-targeting autophagy and the MAPK pathway (with either a MEK or an ERK inhibitor) are ongoing in the laboratory, with *in vivo* studies in mouse PDAC models planned, as described above for Task 3. Should these preclinical studies support the potential efficacy of combined inhibition of autophagy and MAPK signaling, this could represent a novel therapeutic

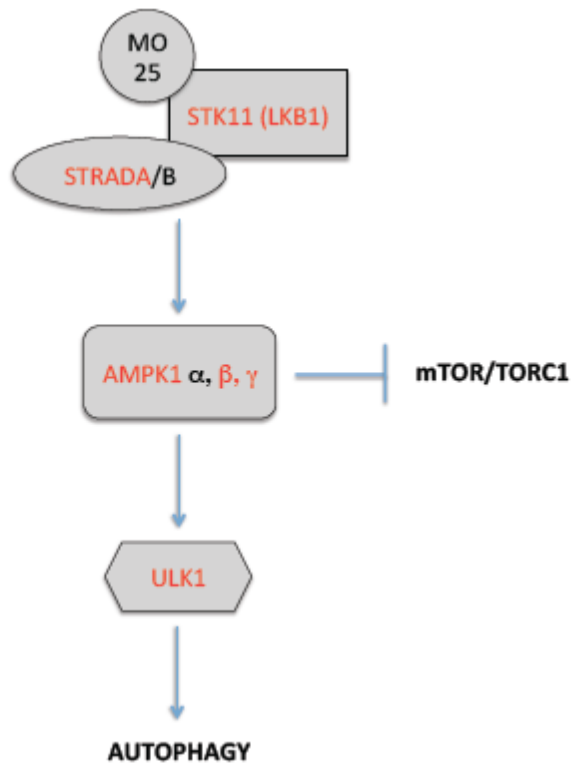

Figure 4: The LKB1-AMPK pathway and autophagy. Pathway members that scored as hits are indicated in red.

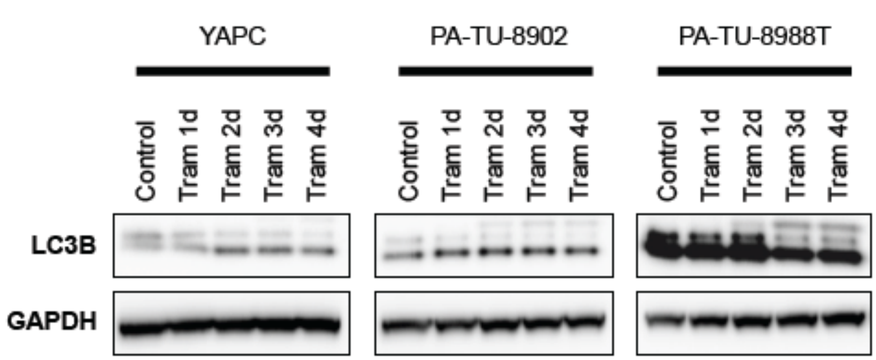

Figure 6: Induction of autophagy in KRAS mutant PDAC cells by MEK inhibition. Western blot of KRAS mutant PDAC cells treated with 100nM trametinib for the indicated times. Induction of autophagy is indicated by a shift toward increased intensity of the lower LC3B band relative to the upper band following treatment.

strategy that could be rapidly translated into clinical trials for PDAC patients, as these individual agents are already under active clinical development.

Overall, we anticipate that our findings regarding the ability of feedback reactivation of MAPK signaling to overcome MAPK blockade by MEK inhibitors in KRAS mutant PDAC cells, and our data demonstrating that ERK inhibitors can promote sustained MAPK suppression and improved efficacy, will influence the design of targeted therapy clinical trials in the near term. Our observations regarding the potential efficacy of co-targeting autophagy and the MAPK pathway in KRAS mutant PDAC has the potential to open a new therapeutic approach for these deadly and difficult to treat cancers.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified feedback reactivation of MAPK signaling through increased RAF activity and MEK hyperactivation as a key mechanism of resistance to MEK inhibitors in KRAS mutant PDAC that likely limits the therapeutic benefit of these agents.
- Found that ERK inhibitors are an alternative strategy for MAPK pathway inhibition in KRAS mutant PDAC that are refractory to feedback reactivation of MAPK signaling and can lead to sustained pathway suppression and improved efficacy in KRAS mutant PDAC. These findings suggest that ERK inhibitors should be actively evaluated in future clinical trials as an alternative approach to MEK inhibitors for MAPK inhibition in KRAS mutant PDAC, both alone and in combination with other targeted agents. Discussion to incorporate ERK inhibitors into clinical trials for PDAC patients are currently underway.
- Identified the LKB1/AMPK and autophagy pathway as a promising clinical target for co-inhibition with the MAPK pathway in KRAS mutant PDAC.

CONCLUSION:

To devise novel targeted therapy combinations for KRAS mutant PDAC, we attempted to develop a large-scale *in vivo* shRNA-drug screen to identify new gene targets that, when inhibited, cooperate with MEK inhibitors to exert anti-tumor activity in KRAS mutant PDAC. Overall, we found that, while an *in vivo* screening strategy may hold potential benefits, the feasibility of a large-scale *in vivo* screening strategy in PDAC with presently available technologies is limited, though future efforts to integrate newer screening technologies to facilitate *in vivo* screening are warranted. Importantly, despite these technical difficulties, our efforts identified feedback reactivation of MAPK signaling through increased RAF activity and MEK hyperactivation as a key mechanism of resistance to MEK inhibitors in KRAS mutant PDAC that likely limits the therapeutic benefit of these agents. Conversely, we found that ERK inhibitors are an alternative strategy for MAPK pathway inhibition in KRAS mutant PDAC that are refractory to feedback reactivation of MAPK signaling and can lead to sustained pathway suppression and improved efficacy in KRAS mutant PDAC. ***Since MEK inhibitors are currently the main class of MAPK pathway inhibitors being evaluated in clinical trials for PDAC and other KRAS mutant cancers and are the backbone for many novel targeted combinations currently in clinical trials, this finding has major implications for targeted therapy strategies for PDAC and other KRAS mutant cancers and suggests that ERK inhibitors should be actively explored in clinical trials for PDAC and other KRAS mutant cancers and could represent a superior backbone for potential therapeutic combinations for these cancers. Discussions to incorporate ERK inhibitors into future targeted therapy trials for PDAC patients are currently underway.*** Our efforts have also identified co-targeting of autophagy and MAPK signaling as a novel therapeutic approach for KRAS mutant PDAC, which could allow the combination of these two promising individual approaches that are each currently in clinical trials for KRAS mutant PDAC. Overall, we anticipate that these findings will impact the development of new clinical trials of novel targeted therapy combinations for PDAC patients in the near term.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Manuscript in preparation defining the role of feedback reactivation of MAPK signaling in overcoming the effect of MEK inhibitors and outlining the potential role of ERK inhibitors as an alterative therapeutic strategy for MAPK blockade.

We expect that our continued efforts defining the role of co-targeting autophagy and MAPK signaling to produce a high impact manuscript within the next 12 months.

INVENTIONS, PATENTS, AND LICENSES:

Nothing to report

REPORTABLE OUTCOMES:

Manuscripts in preparation, as above.

OTHER ACHIEVEMENTS:

Nothing to report

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APPENDICES:

None